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Endomannosidase undergoes phosphorylation in the Golgi apparatus

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Endomannosidase undergoes phosphorylation in the Golgi apparatus

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Glucose residues from *N*-linked oligosaccharides are removed by glucosidases I and II in the endoplasmic reticulum (ER) or by the alternate endomannosidase pathway in the Golgi apparatus. Our morphological analysis demonstrates that recombinant rat endomannosidase exhibited a *cis*- and *medial*-Golgi localization alike the endogenous enzyme and its ER to Golgi transport is COP II mediated. Recombinant endomannosidase undergoes a posttranslational modification, which is not related to *N*- or *O*-glycosylation. A shift in molecular mass of recombinant endomannosidase was observed upon phosphatase digestion but not for ER-retained CHO cell endomannosidase. Furthermore, immunoprecipitation of ³⁵S- and ³³P-labeled endomannosidase expressed in CHO-K1 cells suggests that recombinant endomannosidase undergoes phosphorylation. Substitution of the single cytoplasmic threonine residue of rat endomannosidase by either an alanine or valine residue resulted in the same posttranslational modification alike the wild-type enzyme. The subcellular localization and the *in vivo* activity of the mutant endomannosidase were not affected. Thus, endomannosidase phosphorylation is occurring in luminal sequences. Modification was prevented when endomannosidase was synthesized using reticulocyte lysates in the presence of canine microsomes. Treatment of cells with brefeldin A blocked the posttranslational modification of endomannosidase, suggesting that phosphorylation is occurring in the Golgi apparatus, the residence of endomannosidase.

Keywords: endomannosidase/glucosidase II/Golgi apparatus/*N*-glycosylation/phosphorylation

Introduction

Glucose residues from asparagine-linked Glc₃Man₉GlcNAc₂ oligosaccharides are removed by trimming reactions of glucosidases, which are localized in the endoplasmic reticulum (ER). Their action is important for the biosynthesis of *N*-linked oligosaccharides as well as for the quality control of glycoprotein folding (Parodi 2000; Roth et al. 2002; Helenius and Aebi 2004). Furthermore, the same oligosaccharide is used as a

substrate by endo- α -mannosidase, which provides an alternate glucosidase-independent pathway of glucose trimming in the Golgi apparatus (Lubas and Spiro 1987; Spiro et al. 1997; Spiro 2000; Zuber et al. 2000; Roth et al. 2003).

Endomannosidase cleaves the α 1,2 linkage between the glucose-substituted mannose and the remainder mannose branch of *N*-glycans. Beside tri-glucosylated oligosaccharides, di- and mono-glucosylated oligosaccharides are also processed by the enzyme, even with truncated mannose chains, which are poor substrates for glucosidase II (Lubas and Spiro 1987, 1988; Moore and Spiro 1992; Spiro et al. 1997). This unique endoglycosidase carries out a substantial amount of de-glucosylation *in vivo* as demonstrated by the processing of glycoproteins in HepG2 cells (Weng and Spiro 1996). In glucosidase II-deficient Phar2.7 cells (Reitman et al. 1982) or in glucosidase I-deficient CHO Lec23 cells (Ray et al. 1991; Hong et al. 2004), the processing activity of endomannosidase is essential for the maturation of oligosaccharides to hybrid and complex types. Remarkably, endomannosidase acts on both correctly folded and misfolded proteins. Thus, it provides a back-up mechanism for protein *N*-glycosylation in the Golgi apparatus (Torossi et al. 2006).

Endomannosidase activity is detectable in vertebrate tissues and in a range of animal and human cell lines (Dairaku and Spiro 1997; Spiro et al. 1997; Dong et al. 2000; Zuber et al. 2000). Cloning of endomannosidase from various species (Spiro et al. 1997; Hamilton et al. 2005; Torossi et al. 2007) revealed a high interspecies amino acid sequence identity (Torossi et al. 2007). Recently, we showed that endomannosidase is an integral type II membrane protein (Stehli et al. 2008). Moreover, the first 25 N-terminal amino acids, comprising the cytoplasmic tail and the transmembrane domain are sufficient for Golgi localization of the enzyme (Stehli et al. 2008). In contrast, glucosidase II is ER-retained by the β -subunit carrying the C-terminal KDEL retrieval sequence (Trombetta et al. 1996; Arendt and Ostergaard 1997; D'Alessio et al. 1999). Hence, distinct mechanisms ensure that the glucose-trimming enzymes are located in different compartments (Zuber et al. 2000; Roth 2002) and fulfill specific functions.

Glycoproteins bearing monoglucosylated oligosaccharides will be bound by the chaperone-like lectins calnexin or calreticulin in the ER and released after deglucosylation by glucosidase II (Roth et al. 2008). On the other hand, by the action of Golgi-localized endomannosidase, the Man₈₋₅GlcNAc isomer A is generated which is no more a substrate for re-glucosylation by UDP-glucose:glycoprotein glucosyltransferase (Parodi 2000). As a consequence, the enzymatic activity of endomannosidase in the ER must be inhibited allowing a quality control of glycoprotein folding and assembly by the calnexin/calreticulin cycle (Helenius and Aebi 2004).

In the present study, we report the biosynthesis of endomannosidase as well as its ER to Golgi transport. Furthermore, we

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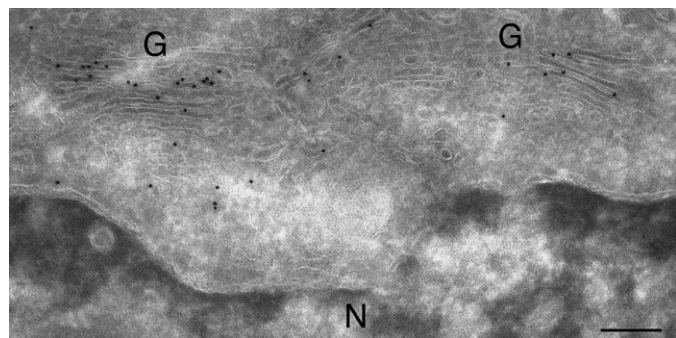


Fig. 1. Recombinant rat endomannosidase is localized in the Golgi apparatus. Immunogold labeling for myc-tagged rEndo in the *cis*- and *medial*-Golgi cisternae and in transport vesicles. N nucleus; G Golgi apparatus. Bar 100 nm.

demonstrate that endomannosidase undergoes phosphorylation in the Golgi apparatus.

Results

ER to Golgi transport of endomannosidase is COP II mediated

Endomannosidase represents a glucose trimming endoglycosidase in the Golgi apparatus and in pre-Golgi intermediates (Zuber et al. 2000). Double immunofluorescence showed that recombinant myc-tagged rat endomannosidase (rEndo) expressed in CHO-K1 cells and in clone 9 hepatocytes, respectively, retained its inherent Golgi localization (Torossi et al. 2007; Stehli et al. 2008). By immunoelectron microscopy, the recombinant enzyme was found in the *cis*- and *medial*-Golgi apparatus and additionally in vesicles (Figure 1). Thus, the recombinant rEndo exhibited a similar subcellular distribution as the endogenous enzyme (Zuber et al. 2000). In general, transport between ER and Golgi apparatus is mediated by COPII-coated vesicles (Hughes and Stephens 2008). We investigated whether recombinant rEndo, expressed in CHO-K1 cells, is a cargo in COP II transport vesicles. Indeed, immunoprecipitated sec23 protein complexes contained recombinant rat endomannosidase (rEndo) or rEndo lacking the positively charged amino acids in the cytoplasmic tail (rEndoR₅₋₇I) (Stehli et al. 2008) (Figure 2). Hence, the transport of myc-tagged rEndo from ER to Golgi appears to occur in COP II-vesicles.

Rat endomannosidase is a nonglycosylated protein

To study the biosynthesis of the enzyme, we expressed the myc-tagged rEndo in CHO-K1 cells as well as in clone 9 rat hepatocytes. Immunoprecipitated endomannosidase from metabolically labeled cells showed a time-dependent increase in molecular mass. Initially the enzyme was synthesized as a 53 kDa protein (Figure 3). After a chase period of 30 min, three bands with higher molecular mass were observed indicating that rEndo undergoes a posttranslational modification. After 2 h chase, the conversion was completed yielding mature rat endomannosidase (rEndo_m) with molecular mass of 58 and 60 kDa. Both bands were detectable even after chase periods of up to 24 h (data not shown). When we expressed the recombinant enzyme in clone 9 hepatocytes the same increase in molecular mass was observed. Previously, no consensus Asn-linked glycosylation

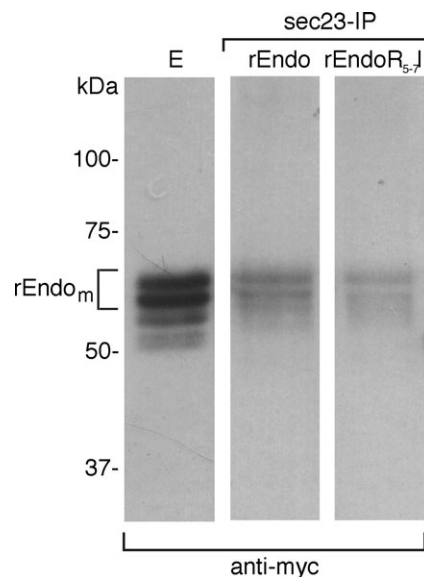


Fig. 2. ER to Golgi transport of endomannosidase is COP II-mediated. Immunoblot analysis of extracts from CHO-K1 cells expressing myc-tagged rEndo (E). Immunoprecipitated sec23-protein vesicles from CHO-K1 cells contain myc-tagged rat endomannosidase (rEndo) or myc-tagged rat endomannosidase R₅₋₇I (rEndo R₅₋₇I). rEndo_m; mature forms of rat endomannosidase.

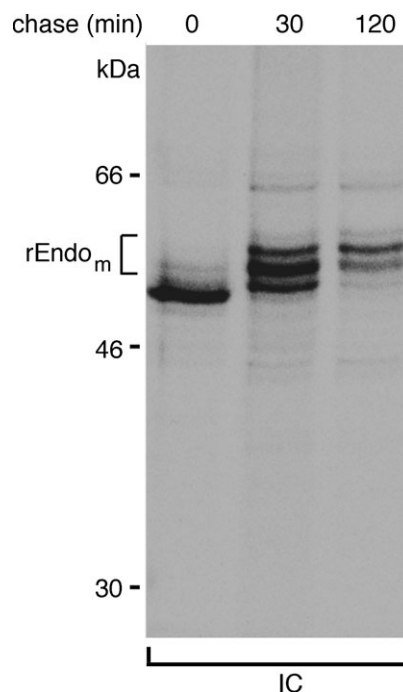


Fig. 3. Rat endomannosidase undergoes a posttranslational modification. CHO-K1 cells expressing myc-tagged rEndo were pulsed for 20 min and chased for the time period indicated. Cell lysates (IC) were immunoprecipitated with anti-myc antibodies and analyzed by 8% SDS-PAGE/fluorography. The molecular mass of recombinant rEndo increased time dependently from 53 kDa (0 min) to 58 kDa and 60 kDa (120 min), respectively. The latter two represent the mature forms of rat endomannosidase, rEndo_m.

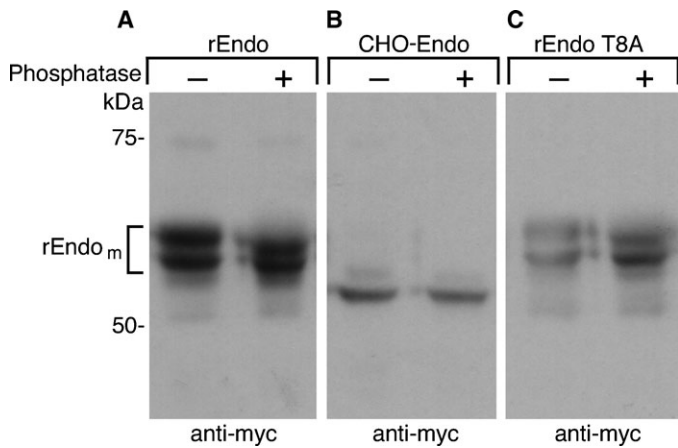


Fig. 4. Rat endomannosidase is phosphorylated. Immunoblot analysis of CHO-K1 cell extracts expressing Golgi-localized myc-tagged rat endomannosidase (A), ER-localized myc-tagged CHO cell endomannosidase (B), or Golgi-localized myc-tagged Thr8Ala-rat endomannosidase (C) in the absence (–) or presence (+) of phosphatase. Phosphatase treatment results in a shift of Golgi-localized rat endomannosidases whereas ER-localized CHO cell endomannosidase is not influenced. rEndo_m, mature forms of rat endomannosidase.

sites were identified in the cloned enzyme (Hamilton et al. 2005; Torossi et al. 2007). Not unexpected, Endo H or PNGase F treatment of mature recombinant rEndo from CHO-K1 cells or from clone 9 hepatocytes did not reduce its molecular mass (data not shown). No evidence for the presence of serine/threonine-linked oligosaccharide side chains on rEndo was obtained by lectin blot analysis using the *Vicia villosa* isolectin B₄, the *Sophora japonica*, the *Dolichos biflorus*, the *Wistaria floribunda*, and the *Amaranthus caudatus* lectins (Goldstein and Poretz 1986). Furthermore, treatment of CHO-K1 cells expressing rEndo with the *O*-glycosylation inhibitor benzyl-GalNac or benzyl-Gal-GalNac (Ramachandran and Peterkofsky 1997) did not result in a shift in the molecular mass of recombinant rEndo suggesting that no elongation of *O*-glycosyl chains occurred (data not shown). Thus, the posttranslational modification of rEndo is not related to *N*- or *O*-linked glycosylation.

Rat endomannosidase is phosphorylated

To further analyze the nature of the posttranslational modification, recombinant rEndo and CHO endomannosidase (Torossi et al. 2007) were subjected to phosphatase treatment. A shift in molecular mass was observed for rEndo (Figure 4A and C) but not for CHO endomannosidase (Figure 4B) upon such a treatment. Thus, we concluded that rEndo is phosphorylated either on a single cytoplasmic threonine or on luminal serine, threonine, or tyrosine residues (Torossi et al. 2007). To substantiate this finding, we expressed both rEndo and CHO endomannosidase in CHO-K1 cells and labeled these cells for 5 h with ³³P (Figure 5). Immunoprecipitated rEndo was labeled with ³³P, indicating that it is modified by phosphate residues (Figure 5A). In contrast, no ³³P incorporation was observed for the ER-retained CHO endomannosidase (Figure 5B). Furthermore, no increase in molecular mass was observed when either rEndo or rEndo lacking its transmembrane domain (ΔTMD-ratEndo) (Stehli et al. 2008) was synthesized with an in vitro transcription/translation system in the presence of canine microsomes

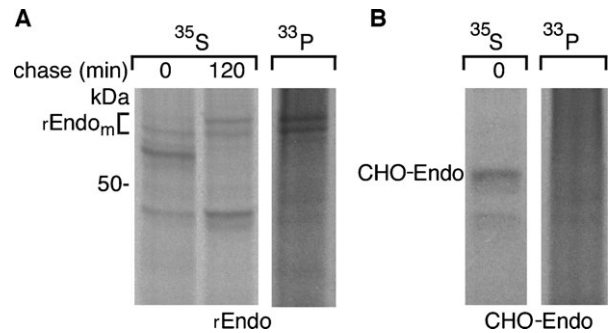


Fig. 5. Only Golgi-localized rat endomannosidase became phosphorylated. CHO-K1 cells stably expressing either Golgi-localized myc-tagged rEndo (A) or ER-localized CHO cell endomannosidase (B) were labeled either with 100 μCi/mL ³⁵S or with 700 μCi/mL ³³P (see *Material and methods*). Myc-tagged endomannosidases were immunoprecipitated from cell lysates and analyzed by 8% SDS-PAGE/fluorography.

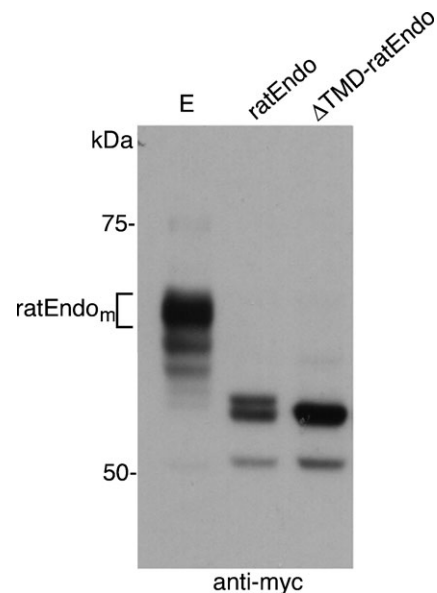


Fig. 6. Posttranslational modification of rat endomannosidase occurs during passage along the secretory pathway. Immunoblot analysis of extracts from CHO cells expressing myc-tagged rat endomannosidase (E) of rat endomannosidase (ratEndo) and of rat endomannosidase lacking its transmembrane domain (ΔTMD-ratEndo), both synthesized in vitro using a reticulocyte lysate system in the presence of canine microsomes. ratEndo_m, mature form of rat endomannosidase.

(Figure 6). However, in vitro synthesis of human α1-antitrypsin yielded EndoH-sensitive species indicating the occurrence of posttranslational modification (data not shown). Similarly, the two bands observed during synthesis of rEndo (Figure 6) might be due to the cleavage of the signal peptide. Thus, we concluded that the posttranslational modification of rat endomannosidase occurs during its passage along the secretory pathway. In order to substantiate that phosphorylation of rEndo might occur in the Golgi apparatus, we used the drug brefeldin A which causes the flow back of *cis*-, *medial*-, and *trans*-cisternae of the Golgi into the ER while leaving the *trans*-Golgi network intact (Lippincott-Schwartz et al. 1989). Treatment of CHO-K1 cells

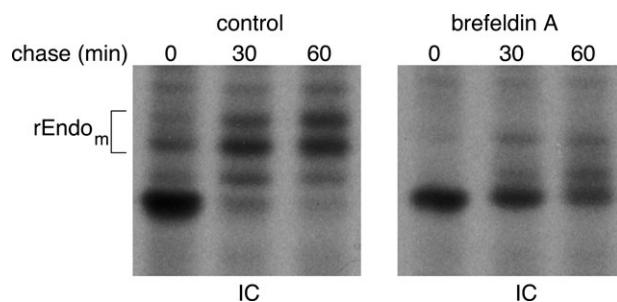


Fig. 7. Brefeldin A prevents the maturation of endomannosidase. CHO-K1 cells expressing Golgi-localized myc-tagged rEndo were preincubated or not for 1 h with brefeldin A (2.5 μ g/mL), pulsed for 20 min with 35 S-methionine, and chased for the time periods indicated. Cell lysates (IC) were immunoprecipitated with anti-myc antibodies and analyzed by 8% SDS-PAGE/fluorography. Brefeldin A blocked the formation of the 58 kDa and 60 kDa mature form of endomannosidase (rEndo_m).

with BFA abolished the shift in molecular mass of rEndo (Figure 7). Hence, phosphorylation occurred in the Golgi apparatus, the residence of endomannosidase.

Rat endomannosidase is phosphorylated in its luminal domain

By site-directed mutagenesis we replaced the single threonine at position 8 in the cytoplasmic tail rEndo by either a valine or alanine residue and expressed the mutant proteins in CHO-K1 cells. Western blot analysis of CHO-K1 cell extracts showed that both rEndo T8A and T8V undergo the same posttranslational modification alike the wild-type enzyme (Figure 4C). Phosphatase treatment of rEndo T8A (Figure 4C) or of rEndo T8V (data not shown) resulted in a shift in molecular mass alike the wild-type enzyme (Figure 4A) suggesting that the single threonine residue in the cytoplasmic tail of rEndo might not be phosphorylated. Double confocal immunofluorescence showed a Golgi localization for rEndo T8A (Figure 8A–C) and for rEndo T8V (data not shown). Furthermore, both mutant rat endomannosidases exhibited fully enzymatic activity (Figure 8D) in processing the *N*-linked oligosaccharides of α 1-antitrypsin (Torossi et al. 2007).

Discussion

In the present study, we showed that recombinant rat endomannosidase (rEndo) exhibited an inherent Golgi localization\alike the endogenous enzyme (Zuber et al. 2000; Stehli et al. 2008; present study). Thus, the recombinant enzyme seems to be properly folded in order to exit the endoplasmic reticulum (ER) (Parodi 2000; Roth et al. 2002; Helenius and Aeby 2004) whereas recombinant CHO cell endomannosidase was ER retained. Recently, we established the molecular defect in CHO cell endomannosidase causing its mislocalization and reduced in vivo activity namely a single Trp188/Cys substitution (Torossi et al. 2007). The export of proteins from the ER is a highly selective process involving COP II transport vesicles (Hughes and Stephens 2008). Indeed, our present biochemical data demonstrate that recombinant rEndo, a type II membrane protein, is a cargo of the COP II complex. Therefore, we conclude that ER to Golgi transport of endomannosidase is COP II-mediated. Several amino acid motifs such as di-acidic, di-hydrophobic,

or di-aromatic sequences were identified to serve as ER export signals. Recently, another type of ER export signal has been described consisting of di-basic amino acids, located proximal to the transmembrane region of Golgi-resident glycosyltransferase. The interaction of the COP II component Sar1p with the di-basic motif seems to be a critical selection of type II membrane proteins as cargo of COP II transport vesicles along the secretory pathway (Giraudo and Maccioni 2003). Cloning of endomannosidase from various species (Hamilton et al. 2005; Torossi et al. 2007) showed that they share as a common feature a cluster of arginine residues in their cytoplasmic tail suggesting that these positively charged amino acids might act as an ER export signal. However, substitution of these amino acid residues by neutral amino acids did not prevent Golgi localization (Stehli et al. 2008; present study). Thus, not only the positively charged amino acids but also structural information of the amino acid side chain influences the ER to Golgi transport competence.

The transport of proteins from the ER to the Golgi apparatus is often accompanied by various posttranslational modifications such as glycosylation, phosphorylation, or sulfation. Indeed, our biochemical analysis demonstrated that recombinant rEndo undergoes a posttranslational modification, which was not related to *N*- or *O*-glycosylation. Phosphatase treatment and in vitro phosphorylation experiments showed that the enzyme is phosphorylated on luminal Ser, Thr, or Tyr residues. However, alkaline phosphatase treatment did not completely abolish the increase in molecular mass. Thus, we conclude that rEndo undergoes phosphorylation beside additional modifications, which remain to be identified. It is well known that various secretory proteins with different biological functions become phosphorylated along the secretory pathway (Wang and Williams 1982; Oetting et al. 1986; Gaut and Hendershot 1993; Swift 1996). Amongst Golgi-localized glycosyltransferases, isoforms of α 2,6-sialyltransferase (ST6Gal-I) are phosphorylated on Ser and Thr residues in their luminal sequences in various cell types (Ma et al. 1999), whereas Ser residues within the cytosolic tail of β 1,4-galactosyltransferase 1 (GalT) are phosphorylated (Strous et al. 1987).

The fact that ER retained CHO cell endomannosidase (Torossi et al. 2007) failed to undergo phosphorylation together with data obtained from our in vitro transcription/translation system suggested that rat endomannosidase phosphorylation occurred in a past ER compartment. Using the drug brefeldin A (Fujiwara et al. 1988; Lippincott-Schwartz et al. 1989), we established the Golgi apparatus as the site of phosphorylation of rEndo. However, we cannot exclude the possibility that rEndo undergoes this posttranslational modification in COP II vesicles. Actually, kinase activities were not only identified in the lumen of the ER (Hendershot et al. 1988), but also in the Golgi apparatus (West and Clegg 1984; Rosa et al. 1992). Moreover, Hirschberg and colleagues (Capasso et al. 1989) identified a functional ATP transporter in the Golgi membrane. Recently, a protein kinase, which phosphorylates serine or threonine residue within atypical cadherin, was identified in the Golgi apparatus (Ishikawa et al. 2008). Thus, our work on rEndo provides additional evidence that phosphorylation of secretory proteins does not occur exclusively in the ER but also in the Golgi apparatus.

Many functions have been attributed to the phosphorylation of proteins. Cell surface localization and function of GalT was influenced by this posttranslational modification (Hathaway et al. 2003). For ST6Gal-I, it was hypothesized that phosphorylation

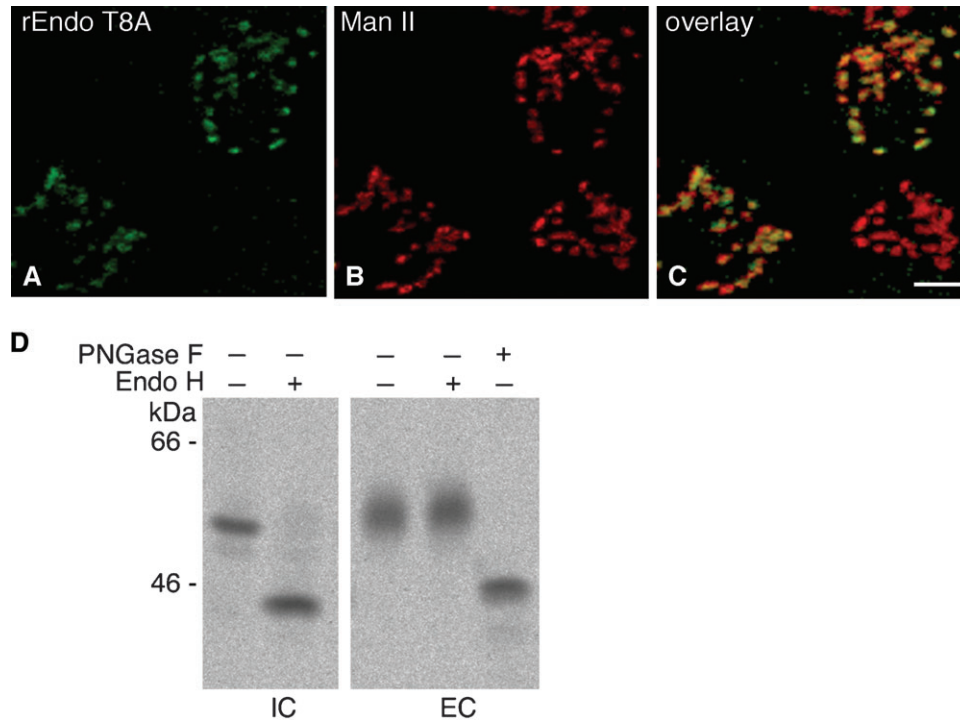


Fig. 8. Engineered rat Endo T8A expressed in CHO-K1 cells exhibited Golgi localization. Confocal double immunofluorescence for myc-tagged rEndo T8A (A) and Golgi mannosidase II (B) showed codistribution (C). Bar, 10 μm. Processing of immunoprecipitated α1-antitrypsin by rEndo T8A from CHO Lec23 cells was analyzed (D). The oligosaccharides of intracellular α1-antitrypsin (IC) were Endo H sensitive, whereas oligosaccharides of secreted α1-antitrypsin (EC) were fully processed as indicated by Endo H resistance and PNGase F-sensitivity.

controls the trafficking to and/or cleavage within the late Golgi apparatus or a post-Golgi compartment (Ma et al. 1999). Furthermore, phosphorylation of a single Ser residue in aquaporin 2 by protein kinase A serves as a key signal for its insertion into the plasma membrane in renal cells (Procino et al. 2003). Control of enzymatic activity or regulation of a protein half-life, as observed for many protein kinases, might be another function of phosphorylation (Hunter 2007). The functional importance of phosphorylation on endomannosidase remains to be investigated in further studies. However, we speculate that the enzymatic activity of Golgi endomannosidase might be controlled by phosphorylation. Finally, one also has to consider that a non-functional phosphorylation can occur (Lienhard 2008).

Material and methods

Antibodies and reagents

The mouse monoclonal anti-myc antibody was from Upstate (Milton Keynes, UK), the polyclonal anti-myc-, the polyclonal anti-sec23, and the polyclonal anti-COP II antibody from Abcam (Cambridge, UK). Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Protease inhibitor tablets, Endo H, PNGase F, and alkaline phosphatase from calf intestine were purchased from Roche Diagnostics (Rotkreuz, Switzerland); TNT coupled reticulocyte lysate systems and canine microsomes from Promega (Wallisellen, Switzerland); pcDNA6/myc-His, competent *E. coli* DH5α cells, lipofectamine 2000, cell culture media and fetal bovine serum

from Invitrogen (Basel, Switzerland). ³⁵S-labeled methionine was purchased from Anawa Trading SA (Wangen, Switzerland) and ³³P from GE healthcare (Otelfingen, Switzerland). Protein A magnetic beads were from Dynal (Hamburg, Germany), biotinylated- or digoxigenin-conjugated lectins, digitonin and all other chemicals of analytical grade from Sigma (Buchs, Switzerland).

Cell lines and transfection

CHO-K1 cells and clone 9 rat hepatocytes were obtained from American Type Culture Collection (Manassas, VA) and grown in Ham's F12 medium containing 10% fetal bovine serum at 37°C in a 5% CO₂ incubator. The CHO Lec 23 cells (Ray et al. 1991; Hong et al. 2004) were kindly provided by Dr. Pamela Stanley (New York, NY) and cultured in Eagle's minimal essential medium (alpha modification) containing 10% fetal bovine serum (FBS). Stable transfections were performed as described previously (Torossi et al. 2007; Stehli et al. 2008).

Site-directed mutagenesis in rat endomannosidase

Mutant rEndo were generated by a PCR-based site-directed mutagenesis strategy as described (Stehli et al. 2008).

Metabolic labeling, immunoprecipitation, and SDS-PAGE

CHO-K1 cells or clone 9 hepatocytes stably expressing myc-tagged rEndo (Torossi et al. 2007; Stehli et al. 2008) were incubated in methionine-free DMEM for 30 min at 37°C in a 5% CO₂ incubator. Afterward, cells were labeled in medium containing 100 μCi/mL ³⁵S-methionine for 20 min and chased

with medium containing cold methionine (1 mM) for the indicated time points. Cells treated with brefeldin A (BFA) were preincubated in medium containing 2.5 $\mu\text{g/mL}$ BFA for 1 h. The same concentration of BFA was maintained throughout the methionine-depletion, labeling, and chase periods. Cells were washed with ice-cold phosphate-buffered saline pH 7.4 (PBS), mechanically removed with a rubber policeman and resuspended in PBS containing protease inhibitors. Proteins were extracted with Triton X-100 (1%, 1 h at 4°C) and cell debris removed by centrifugation. The cell extracts and the corresponding culture mediums were added to protein A magnetic beads conjugated with a rabbit anti-myc antibody and incubated for 16 h at 4°C. Immunoprecipitates were washed with PBS containing 0.1% Triton X-100 and analyzed by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Combined immunoprecipitations/immunoblot analysis

Immunoprecipitations of myc-tagged rEndo were performed as described above. For COPII immunoprecipitations, anti-sec23 antibodies, crosslinked to magnetic beads, were added to digitonin-lysed cells. Immunoprecipitates and CHO cell extracts were analyzed by 8% SDS-PAGE and transferred onto nitrocellulose membranes using a semidry blotting apparatus (Towbin et al. 1979). The nitrocellulose membranes were blocked in PBS containing 1% BSA for 1 h at ambient temperature and incubated with a mouse anti-myc antibody, overnight at 4°C followed by the incubation with the corresponding horseradish-conjugated secondary antibody for enhanced chemiluminescence detection.

Phosphatase digestion, glycosidase treatment, and lectin blotting

For phosphatase digestion, CHO-K1 cell extracts were digested with 2 U alkaline phosphatase for 16 h at 37°C. The samples were analyzed by SDS-PAGE/immunoblotting as described above. Immunoprecipitated myc-tagged rEndo was subjected to Endo H and PNGase F treatment as described previously (Torossi et al. 2006). For lectin blotting, the nitrocellulose membranes were either incubated with digoxigenin-conjugated or biotinylated lectins according to the manufacturer's instructions. Lectin binding was detected by using alkaline phosphatase-conjugated polyclonal sheep anti-digoxigenin Fab' fragments or alkaline phosphatase-conjugated streptavidin. Enzyme activity was revealed by using the nitroblue tetrazolium/X-phosphate substrate system. As control for lectin blotting, nitrocellulose stripes containing immobilized rat megalin was used (Ziak et al. 1999).

In vivo phosphorylation of rat endomannosidase

For labeling of proteins with ^{33}P , CHO-K1 cells expressing myc-tagged rEndo or CHO endomannosidase (Torossi et al. 2007; Stehli et al. 2008) were incubated in DMEM medium without sodium phosphate for 30 min at 37°C in a 5% CO_2 incubator. Cells were labeled in medium containing 700 $\mu\text{Ci/mL}$ of ^{33}P for 5 h at 37°C. Lysis of the cells and analysis of the immunoprecipitates were performed as described above. Radioactivity was visualized by autoradiography or by using a phosphorimager (FUJI Film Corp., Japan).

In vitro transcription and translation

Coupled, nonradioactive in vitro transcription and translation, in the presence or absence of canine microsomes, was performed with 1 μg of Fsp I-linearized pcDNA6/rat- or CHO endomannosidase (Torossi et al. 2007) as a template in a total reaction volume of 25 μL , according to the manufacturer's instructions. As a control, 1 μg of Sca I-linearized pcDNA3/ $\alpha 1$ -antitrypsin (Torossi et al. 2006) was used. At the end, 5 μL of reaction mixtures was analyzed by SDS-PAGE/immunoblotting as described above.

Confocal immunofluorescence microscopy and in vivo endomannosidase assay

Transfected CHO-K1 cells grown as a monolayer on glass cover slips were formaldehyde fixed and saponin permeabilized. Double immunolabeling incubations were performed as described (Stehli et al. 2008). Endomannosidase activity was determined on the basis of the processing of the oligosaccharides of human $\alpha 1$ -antitrypsin (Stehli et al. 2008).

Immunoelectron microscopy

CHO-K1 cells were fixed in 3% paraformaldehyde in PBS, embedded in low-melting agarose, and infiltrated with a sucrose-polyvinyl pyrrolidone mixture. Frozen ultrathin cryosections were prepared according to Tokuyasu (1978, 1980), incubated with a mouse anti-myc antibody followed by a 8 nm gold-labeled secondary antibody according to standard protocols (Roth et al. 1978).

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Conflict of interest statement

None declared.

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